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Single nucleotide polymorphisms and haplotypes in the IL10 region associated with HCV clearance

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Hepatitis C virus (HCV) is an infectious blood-borne pathogen that usually persists as a chronic infection. However, approximately 15% of the time, patients can clear the virus, indicating that host differences could be critical in determining the course of HCV infection. The inflammatory response is crucial to resolving or failing to resolve an acute HCV infection. Some previous reports have implicated interleukin 10 (IL10) polymorphisms with successful anti-HCV therapy and natural viral clearance. We tested 54 single nucleotide polymorphisms (SNPs) in the IL10 region (±300 kb and 24 within the IL10 gene itself), which contains 13 genes including the IL10 immunomodulatory paralogs IL19, IL20, and IL24, for association with HCV clearance vs persistence. SNPs from two haplotype block regions, one at IL10 and the other from IL19/IL20, were associated with HCV clearance in African Americans (91 clearance cases and 183 chronically infected matched controls; P = 0.05–0.002) while with expectation-maximization algorithm-reconstructed haplotypes, these associations remained (P = 0.05–0.002). However, no significant associations were detected in European Americans (108 clearance and 245 chronic). Our results indicate that variants of the immunomodulatory IL10 and IL19/IL20 genes may be involved in natural clearance of HCV in the African-American population.

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Introduction

The hepatitis C virus (HCV), an RNA virus from the *Flaviviridae* family,[1] is the most common chronic blood-borne pathogen in the United States. HCV is transmitted by percutaneous exposure to contaminated blood, perinatal exposure from a mother to her infant, unprotected sexual intercourse, and poor medical care practices in developing nations.[2] Drug use has consistently accounted for a substantial proportion of HCV infections and currently accounts for 60% of HCV transmission while sexual exposures account for up to 20%.[3] There are 3.9 million HCV-infected people in the United States alone, with an estimated prevalence of 1.8%.[3] While the annual incidence of new infections in the United States has decreased more than 80% in the last decade, chronic hepatitis still results in as many as 10 000 deaths per year because of the latency of developing complications.[4] HCV infection is also a common problem worldwide, with at least 170 million people infected.[4] The estimated annual total cost for HCV treatment in the United States in 2003 was between $129 and $514 million. The total cost of treating HCV infection, including pharmaceutical costs and outpatient therapy, was estimated to be $693 million. Overall, the total economic impact of the disease in the United States is between $1 and $1.3 billion per year and is considerably more worldwide.[5,6]

HCV infection sometimes results in clearance (15%),[7] a state that is recognized when HCV RNA cannot be detected in multiple blood samples from a patient with HCV-specific antibodies.[1] HCV clearance is known to occur less often in blacks people, alcohol users, and HIV-infected persons.[7,8] In the chronic state, the infection may progress to cirrhosis with the subsequent development of complications such as ascites, encephalopathy, variceal bleeding, and hepatocellular carcinoma because of the continual inflammatory response to the viral infection. As many as 75% of HCV-positive patients in the United States have the chronic form of HCV infection,[3] most of which will develop chronic hepatitis and progressive fibrosis.[9] Within 10 years, some of the fibrosis patients will develop cirrhosis, while some others will not have notable liver disease during their lifetime.[10]

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A number of studies have indicated the possibility of a positive association between HCV disease outcome and genetic polymorphisms. For example, many immunological proteins may play a role in the response to HCV antiviral treatment: in particular, interferons (IFNs), tumor necrosis factor (TNF), cytokines, and chemokines. Polymorphisms in several genes including *MxA, PKR, OAS*,[11] *HLA, KIR, TNF*,[12] and *MICA* have been shown to be associated with HCV clearance or persistence.[13,14] Studies have indicated that single nucleotide polymorphisms (SNPs) in the *IL10*, *CTLA*, *MxA*, and *LMP7* genes may influence the response to IFN-α treatment in patients with chronic HCV[15–17] along with variants in the HLA.[13,14] The strength of the immune response may influence HCV-mediated fibrogenesis[10] through cytokines that stimulate extracellular matrix deposition such as TNF. However, current genetic studies were unable to identify the link between functional *TNF* SNPs and histological severity or response to antiviral therapy.[18] Conversely, inheritance of the *MCP1* alleles may predispose HCV patients to more severe hepatic inflammation and fibrosis.[19] A recent report has also implicated one *MICA* allele in HCV clearance utilizing the same patient set.[20]

The genetic associations of *IL10* with treatment and the role of inflammation in HCV disease led us to consider the family of interleukin-10 (IL-10)-related cytokines that has several cellular paralogs, including *IL19*, *IL20*, *IL22*, *IL24*, and *IL26*, as candidate genes involved with HCV clearence.[21] IL-10 plays an important part in the regulation of cellular immune responses and in the suppression of proinflammatory cytokine secretion in a number of different cell types. IL-10 is a cytokine synthesis inhibitory factor that balances the TH1 and TH2 immune response, and is involved in many aspects of human disease.[22,23] There is strong evidence for a substantial genetic component in IL-10 production.[24,25] The *IL10* gene has been implicated in the response to a number of diseases such as hepatitis B, pulmonary tuberculosis, herpes zoster, cutaneous malignant melanoma, hepatocellular carcinoma fibrosis, gastric carcinoma, squamous cell carcinoma, inflammatory bowel disease, and HIV.[26–34] The genetic neighbors of *IL10* consisting of *IL19*, *IL20*, and *IL24* also modulate the TH1 and TH2 response, suggesting that these recently discovered loci could also be important in HCV clearance.[35]

A number of studies have examined the *IL10* promoter region SNPs and their associations with HCV susceptibility as well as resistance to antiviral therapy. Current therapy of chronic HCV infection is based on type I IFN-α treatment along with ribavirin.[36] The commercial IFN-α preparations that are largely used for HCV therapy consist of IFN-α2a or IFN-α2b subtypes.[37] Some of these studies indicate that there is a positive *IL10* association with susceptibility to chronic hepatitis C infection and resistance to combined antiviral therapy[38–41] and rapid fibrosis[39] while in others the association between *IL10* promoter region SNPs and viral clearance or persistent infection or severity of disease was not found.[42–44] These results further indicated the importance of evaluating HCV clearance in a large set of patients for *IL10*, its neighboring paralogs, and other genes in the region.

We set out to provide a comprehensive evaluation of the association between *IL10* and its neighboring paralogs with HCV clearance *vs* persistence among infected individuals from multiethnic cohorts while evaluating linkage disequilibrium (LD) in the region. Several polymorphisms located close to or within the *IL10* gene are associated with transcription levels[45] and the nearby flanking genes should be examined for their potential impact on disease. The best-studied SNPs in the *IL10* gene are in the promoter positions –1082 (rs1800896), –819 (rs3021097), and –592 (rs1800872).[46] In the present study, we used 32 SNPs in the *IL10* gene itself and an additional 41 from the surrounding region of approximately 300 Kb to each side, which includes the genes *FNBP2, IKBKE, RASSF5, LGTN, DYRK3, MAPKAPK2*, *TOSO, PIGR, FKSG87*, and *SARG* along with the *IL10* paralogs *IL19*, *IL20*, and *IL24* (Figure 1). Then, we chose SNPs that indicated a significant association with HCV

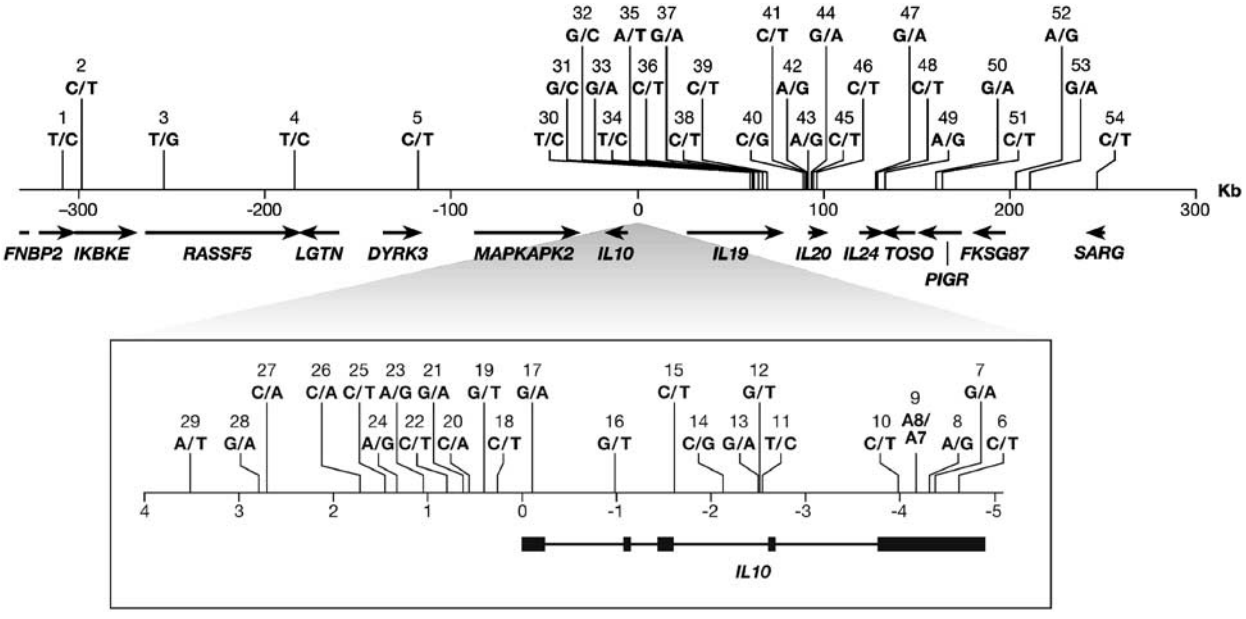

Figure 1 Locations and variation of SNPs in and around the *IL10* gene on chromosome 1 with SNPs numbered as shown in Table 1.

clearance, and tested them further by assessing different genetic models. The corresponding haplotypes within the haplotype blocks were estimated and examined for an association with HCV clearance as well.

Results

The effects of *IL10*, its paralogs, and genetic polymorphisms in the flanking region were assessed for HCV clearance by examining 54 polymorphic SNPs (Table 1). The subjects examined were either African Americans (91 clearance cases and 183 chronically infected matched controls) or European Americans (108 clearance cases and 245 chronically infected individuals) matched two for one on ethnicity, HIV status, and gender.

In the single SNP analyses, there were several alleles, genotypes, and haplotypes that had a significant association with HCV clearance in African Americans while no significant associations were observed for alleles or haplotypes in European Americans (Table 2). In the allelic tests, the strongest associations were seen with three SNPs in *IL10*, rs6703630, rs6693899, and rs3024498 ($P = 0.03$–0.004), as well as the SNPs in *IL19*, rs2243191, and *IL20*, rs1400986, rs3024517, and rs2232360 ($P = 0.05$–0.01). Analyses of these seven loci remained significant in the genotypic tests for both the additive and/or dominant tests with the addition of rs2981573 (Table 2; $P = 0.05$–0.002). The two classical SNPs from the proximal part of the *IL10* promoter region, rs1800896 (−1082) and rs1800872 (−592), previously reported to be associated with the response to HCV infection and treatment that define the GCC, ACC, and ATA haplotypes,[46] did not show a significant association with clearance in either of the racial groups for these tests ($P > 0.2$). On the other hand, two out of three loci in the distal part of the promoter reported to be associated with the levels of IL-10 production (rs6693899 and rs6703630)[45] were significantly associated with the chronic outcome of HCV infection in African Americans (Table 2; $P = 0.05$–0.004).

Two regions of LD were apparent, with the first one encompassing *IL10* and a second one that included *IL19* and *IL20* (Figure 2). We analyzed these blocks of SNPs with the EM algorithm implementation in Proc Haplotype[47] to estimate haplotypes using loci that were associated with HCV ($P < 0.05$) in the previous analyses. While the proximal SNPs (−1082 and −592) did not show a significant association with clearance, due to their role in the *IL10* expression and previous reports of association with this disease and numerous others, they are included in both the single SNPs and haplotype analyses. Overall, there were 10 haplotypes extending over the *IL10* region and six over the *IL19*/*IL20* region that were evaluated in clearance and chronic groups of European Americans and African Americans (Table 3).

As in the single SNP analyses, almost all of the haplotype-based associations observed were seen in African Americans for the allelic, additive, and dominant analyses (Table 3). Only one haplotype in the *IL10* region, AAGCG, was associated with HCV clearance, and the association was observed in allelic, codominant, and dominant models (odds ratio (OR) = 0.47–0.48, $P = 0.03$–0.01). There were two haplotypes in the *IL19*/*IL20* region in African Americans significantly associated with HCV clearance, the depleted CTGAAC (OR = 0.56–0.59, $P = 0.05$–0.04) and the enriched TCAGGC (OR = 1.93–2.7, $P = 0.01$–0.002). In addition, as a test of association of extended haplotypes in the promoter region with HCV,[38–41] we analyzed the association of well-studied proximal[46] and distal haplotypes[45] and HCV clearance (Table 4). These results indicated that distal haplotypes AAA (OR = 0.38, $P = 0.002$–0.003), and TGC (OR = 1.52, $P = 0.04$–0.03), but not proximal haplotypes in *IL10* are associated with clearance of HCV in the African-American population.

Discussion

Understanding the genetic basis of host-limiting infection of HCV (clearance without any HCV-specific therapy) provides a direct approach to finding clues to treatment of this chronic infection. Given previous reports of the role of *IL10* variants in both HCV therapy and clearance, we sought to evaluate the role of genetic variants at this gene and its neighbors. We examined a total of 274 African Americans (91 clearance cases and 183 chronically infected matched controls) and 353 European Americans (108 clearance and 245 chronic). In essentially African Americans only, there were several alleles, genotypes, and haplotypes in *IL10* and the *IL19*/*IL20* genes that had significant associations with HCV clearance in African Americans. Most of these SNPs have not been associated with HCV outcome in the past, which opens new avenues for candidate gene testing for this disease.

An examination of the *IL10* region found at least two large haplotype blocks: one around *IL10* and a second one encompassing *IL19* and *IL20.* These are regions with little evidence for historical recombination and only a limited number of common haplotypes observed.[48] The boundaries of blocks and specific haplotypes were similar across population groups, but additional structure was visually evident when examining r^2 and $|D'|$ in African Americans than European Americans (Figure 2). As expected, African Americans had more common haplotypes,[48] and analyses of some of these indicated possible associations with HCV (Table 3, $P = 0.05$–0.002).

Several recent studies suggested that *IL10* polymorphisms may influence HCV outcome in the host[38–41,49] while others have not found associations.[42,43] Generally, these are concentrated on the proximal *IL10* promoter polymorphisms that have been associated with the differential IL-10 expression,[46,50] while other polymorphisms, particularly those in the distal part of the promoter, have also been implicated.[45] More recently, different proximal and distal polymorphism effects on IL-10 production have provided a more complex picture of gene expression.[51] Concurrently, HCV treatment and *IL10* candidate gene studies have suggested an association of extended haplotypes in the promoter region.[38–41] As a test of these hypotheses, we performed a separate analysis using the well-studied proximal[46] and distal haplotypes[45] (Table 4). We did not observe any positive associations between the proximal *IL10* promoter SNPs of −1082 and −592 defining the GCC, ACC, and ATA haplotypes[46] with HCV clearance (Table 4). While the functional control of expression by the −1082 and −592 SNP containing haplotypes is very well supported,[40,41,45,52,53] their

Table 1 SNPs examined — nearby gene, locus names, polymorphism types, location, and minor allele frequencies of SNPs screened in the study of HCV clearance[1]

						European Americans		*African Americans*	
No.	*Gene*	*Locus*[a]	*Polymorphism*	*Position*[b]	*Alleles*[c]	*Clearance (n = 108)*	*Chronic (n = 245)*	*Clearance (n = 91)*	*Chronic (n = 183)*
1	*FNBP2*	rs9242	3′ UTR	−308.44	T/C	0.45	0.46	0.51	0.47
2	*IKBKE*	rs1539243	Syn (Ile→Ile)	−298.05	C/T	0.19	0.14	0.11	0.14
3	*RASSF5*	rs944769	Intronic	−253.76	T/G	0.48	0.49	0.77	0.77
4	*RASSF5*	rs11589	3′ UTR	−183.90	T/C	0.31	0.31	0.32	0.31
5	*intergenic*	rs13208	Intergenic	−117.68	C/T	0.42	0.38	0.44	0.42
6	*IL10*	rs6687786	3′ UTR	−4.62	C/T	0	0	0.09	0.06
7	*IL10*	rs6697497	3′ UTR	−4.38	G/A	0	0	0.09	0.07
8	*IL10*	**rs3024498**	3′ UTR	−4.31	A/G	0.28	0.28	0.09	0.17
9	*IL10*	*IL-10* 8161	3′ UTR	−4.17	A8/A7	0	0	0.10	0.07
10	*IL10*	rs3024496	3′ UTR	−3.98	C/T	0.50	0.49	0.60	0.57
11	*IL10*	rs3024509	Intronic	−2.54	T/C	0.07	0.07	0.02	0.01
12	*IL10*	*IL-10* 6520	Intronic	−2.52	G/T	0	0	0.01	0
13	*IL10*	rs3024494	Intronic	−2.49	G/A	0	0	0.06	0.05
14	*IL10*	rs1878672	Intronic	−2.13	C/G	0.47	0.47	0.24	0.31
15	*IL10*	rs1554286	Intronic	−1.61	C/T	0.17	0.17	0.40	0.36
16	*IL10*	rs1518110	Intronic	−0.98	G/T	0.22	0.21	0.42	0.38
17	*IL10*	*IL-10* 4099	Promoter	−0.10	G/A	0	0	0	0.01
18	*IL10*	rs5743625	Promoter	0.28	C/T	0.01	0.01	0.01	0
19	*IL10*	rs3024489	Promoter	0.41	G/T	0	0	0.03	0.03
20	*IL10*	**rs1800872**	Promoter	0.57	C/A	0.22	0.23	0.43	0.38
21	*IL10*	rs1800895	Promoter	0.63	G/A	0.01	0	0	0.01
22	*IL10*	rs1800871	Promoter	0.80	C/T	0.23	0.23	0.42	0.38
23	*IL10*	**rs1800896**	Promoter	1.06	A/G	0.50	0.50	0.32	0.36
24	*IL10*	rs1800893	Promoter	1.33	A/G	0.49	0.50	0.65	0.62
25	*IL10*	rs5743624	Promoter	1.47	C/T	0.05	0.03	0.05	0.03
26	*IL10*	rs5743623	Promoter	1.73	C/A	0.01	0.01	0.01	0.03
27	*IL10*	**rs6693899**	Promoter	2.71	C/A	0.37	0.38	0.27	0.39
28	*IL10*	**rs6703630**	Promoter	2.80	G/A	0.27	0.29	0.19	0.32
29	*IL10*	rs1800890	Promoter	3.53	A/T	0.39	0.39	0.21	0.28
30	*IL19*	rs2243155	Intronic	60.14	T/C	0	0	0.05	0.05
31	*IL19*	rs2243156	Intronic	60.38	G/C	0.09	0.09	0.18	0.13
32	*IL19*	rs2243158	Intronic	61.80	G/C	0.10	0.09	0.19	0.12
33	*IL19*	rs2243161	Intronic	62.26	G/A	0	0	0.12	0.15
34	*IL19*	rs2243164	Intronic	62.73	T/C	0	0	0.16	0.12
35	*IL19*	rs2243168	Intronic	63.55	A/T	0.09	0.09	0.31	0.27
36	*IL19*	rs2073186	Intronic	64.79	C/T	0.27	0.24	0.39	0.37
37	*IL19*	rs2073185	Intronic	64.89	G/A	0.16	0.12	0.03	0.04
38	*IL19*	**rs2243176**	Intronic	66.61	C/T	0.18	0.14	0.08	0.11
39	*IL19*	**rs2243191**	Non-syn (Ser→Phe)	70.12	C/T	0.25	0.23	0.23	0.15
40	*IL20*	**rs1713239**	Intergenic	91.64	C/G	0.16	0.13	0.04	0.05
41	*IL20*	**rs1400986**	Intergenic	92.85	C/T	0.16	0.18	0.30	0.40
42	*IL20*	**rs3024517**	Intronic	94.41	A/G	0.15	0.17	0.12	0.19
43	*IL20*	**rs2981573**	Intronic	94.74	A/G	0.25	0.21	0.22	0.15
44	*IL20*	**rs2232360**	Intronic	94.82	G/A	0.26	0.22	0.22	0.15
45	*IL20*	**rs1109461**	Intronic	95.96	C/T	0.01	0.01	0.27	0.26
46	*IL20*	**rs1518108**	Intergenic	97.34	C/T	0.42	0.49	0.52	0.58
47	*IL24*	rs3093426	Intronic	127.64	G/A	0.01	0.01	0.24	0.20
48	*IL24*	rs1150258	Non-syn (His→Tyr)	129.07	C/T	0.42	0.50	0.26	0.33
49	*TOSO*	rs188334	Intronic	133.78	A/G	0.41	0.49	0.22	0.20
50	*PIGR*	rs291102	Non-syn (Val→Ala)	160.64	G/A	0.13	0.08	0.89	0.90
51	*PIGR*	rs2275531	Non-syn (Ser→Gly)	163.28	C/T	0.40	0.48	0.20	0.22
52	Intergenic	rs1890865	Intergenic	206.92	A/G	0.04	0.02	0.55	0.56
53	Intergenic	rs1890866	Intergenic	217.06	G/A	0.03	0.05	0.02	0.01
54	*SARG*	rs10877	3′ UTR	246.34	C/T	0.19	0.23	0.31	0.32

The following loci were found to be invariable in the present data set (frequency <1% in both African Americans, and European Americans): rs3024510, rs1518111, rs3024506, *IL10* 3462, *IL10* 2931, *IL10* 2410, *IL10* 1974, and *IL10* 1273 from the IL10 region, and rs2297542, rs1129431, rs1129432, rs110514, rs113528, rs7868, rs2243173, rs3024523, rs291109, rs3093431, rs3093446, and rs2054779 from the surrounding regions.

[a]Loci shown in bold are for $P<0.05$ in the simple allelic χ^2 case–control tests, and followed up in subsequent analysis.

[b]Position (kb) is relative to the *IL10* gene's start of transcription on the genomic sequence.

[c]Minor allele is the second one shown whose frequency is reported in the table.

reported role in natural HCV clearance and persistence[38–41] is likely only at most a mild effect. On the other hand, in the distal region of the promoter, low-production haplotype AAA (Table 4) was associated with the chronic infection only in African Americans (OR = 0.38, $P = 0.003$–0.002), while analysis of the high-production

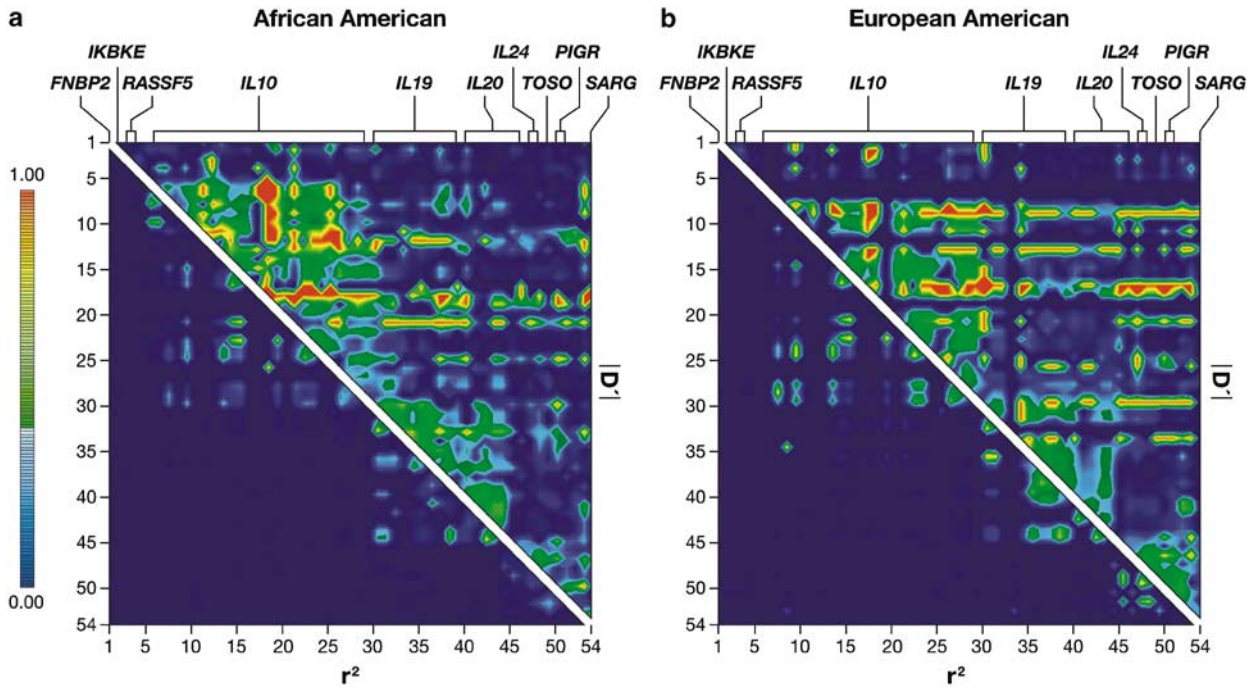

Figure 2 Plot of LD across the *IL10* region. Higher values of r^2 and $|D'|$ represented by the red part of the spectrum indicate elevated levels of LD between the two SNPs plotted on both the *X* and *Y* axes and numbered as shown in Table 1. Two distinct LD groups in the *IL10* and *IL19/IL20* regions are evident from examination of African Americans (**a**) and European Americans (**b**).

haplotype TGC suggests association with HCV clearance (dominant model, OR = 1.52–2.55, $P = 0.04$–0.03).

Overall, our results indicate that selected *IL10* and *IL19/IL20* gene SNPs and haplotypes are involved in clearance of HCV in the African-American population and do not support previous reports of associations in European Americans. This disparity may be due to the ethnic differences, but may also be related to the route of infection. That associations were only seen in African Americans who are largely drawn from an intravenous drug user (IVDU) cohort and not in European Americans largely drawn from hemophiliac cohorts could be due to infectious doses that are lower in needle-borne transmission as compared to blood transfusions. The recent association of HLA and NK cell receptor genes with HCV clearance saw genetic effects at low infectious doses (only in those who did not receive blood products).[54] A similar infectious dose response for CMV with the murine NK cell receptor Ly49 H has also been observed.[55,56] Together with the observation that essentially all significant associations were seen in low-dose IVDUs who happen to be African American in this study, our results support further evaluation of variants at *IL10*, *IL19*, and *IL20* in HCV clearance.

Since in this study we applied allelic tests to 54 different SNPs, our significance levels for *P*-values would have to be lowered to the approximately $P < 0.001$ level to distinguish significant test results from those that appear by chance alone.[56–59] None of the tests of association with HCV clearance were significant after this 'Bonferroni' correction (the lowest value was $P = 0.002$ (Table 2)). However, eight different loci showed significance ($0.05 < P < 0.002$) in African Americans, when we expected fewer (approximately 2.7 with the experiment-wide error rate of 5%). If these 54 tests were independent, such a distribution of *P*-values could not be attributed to random events alone (one-tailed binomial expectations, $P = 0.005$). Thus, we expect that five of these eight $P < 0.05$ loci at *IL10*, *IL19*, and *IL20* reveal biologically significant associations with HCV clearance in African Americans. In contrast, the analysis of European Americans found only one *P*-value < 0.05, which very likely results from multiple testing.

While we cannot point to one genetic variant as a cause in HCV clearance, there is a significant excess of associations with *IL10*, *IL19*, and *IL20* variants. The suggestive results with *IL19* and *IL20* are intriguing, given their newly discovered roles in modulating the TH1 and TH2 response where *IL10* is already known to be intimately involved.[35] Research on larger well-defined cohorts will be needed to test for what are potentially weak effects at *IL10* and *IL19/IL20*.

The association seen with *IL19* is particularly promising, given this gene's involvement in chronic inflammatory diseases through suppressing the TH1 response and inducing TH2.[60] The many associations seen at *IL20*, which is important in the inflammatory response[61] and in enhancing growth of multipotential hematopoietic progenitor cells,[62] suggest that this gene may be important in HCV clearance. *IL10* and its neighboring paralogs (*IL19* and *IL20*) may provide a link between host genetic polymorphisms and HCV clearance through modulating the TH1 and TH2 response in concert. Further research on the functional implications of the *IL10*, *IL19*, and *IL20* genetic polymorphisms and the HCV clearance is needed to clarify mechanisms of action.

Searches with additional candidate genes and novel genetic approaches for HCV clearance can further our understanding of the HCV progression and outcome. Some new approaches, such as mapping by admixture

Table 2 Major allele frequencies and results of conditional logistic regression to examine the association between HCV clearance and selected genotypes in specific genetic models and tests[a]

		European Americans								*African Americans*							
		Allele freq. (%)		*Allelic*[b]		*Codominant*		*Dominant*		*Allele freq. (%)*		*Allelic*		*Codominant*		*Dominant*	
Gene SNP (no.)	*Alleles*[c]	*Clearance (n = 108)*	*Chronic (n = 245)*	*Odds ratio*	*P*	*Relative hazard*	*P*	*Odds ratio*	*P*	*Clearance (n = 91)*	*Chronic (n = 183)*	*Odds ratio*	*P*	*Relative hazard*	*P*	*Odds ratio*	*P*
IL10																	
rs6703630 (28)[d]	G/A	27	29	0.91	0.65	0.9	0.63	1.01	0.96	19	32	0.52	0.004	0.54	0.01	0.5	0.01
rs6693899 (27)	C/A	37	38	0.99	0.95	0.99	0.95	0.93	0.79	27	39	0.58	0.01	0.57	0.01	0.59	0.05
rs1800896 (23)	A/G	50	50	10.05	0.8	0.93	0.71	0.91	0.71	32	36	0.84	0.37	1.22	0.28	1.43	0.2
rs1800872 (20)	C/A	22	23	0.93	0.71	0.93	0.71	0.91	0.71	43	38	1.22	0.27	1.22	0.28	1.43	0.19
rs3024498 (8)	A/G	28	28	1.08	0.69	1.09	0.68	1.24	0.42	9	17	0.53	0.03	0.56	0.04	0.59	0.08
IL19																	
rs2243176 (38)	C/T	18	14	1.27	0.29	1.26	0.3	1.18	0.52	8	11	0.7	0.28	0.73	0.31	0.61	0.17
rs2243191 (39)	C/T	25	23	1.03	0.90	1.03	0.89	1.09	0.72	23	15	1.63	0.04	1.70	0.03	1.99	0.01
IL20																	
rs1713239 (40)	C/G	16	13	1.16	0.55	1.16	0.54	1.17	0.56	4	5	0.87	0.76	0.86	0.76	0.86	0.76
rs1400986 (41)	C/T	16	18	0.96	0.85	0.95	0.84	0.8	0.4	30	40	0.59	0.01	0.62	0.01	0.43	0.002
rs3024517 (42)	A/G	15	17	0.93	0.75	0.92	0.74	0.79	0.41	12	19	0.58	0.04	0.58	0.04	0.55	0.04
rs2981573 (43)	A/G	25	21	1.23	0.29	1.25	0.28	1.35	0.22	22	15	1.56	0.07	1.60	0.06	1.83	0.04
rs2232360 (44)	G/A	26	22	1.16	0.45	1.17	0.44	1.29	0.29	22	15	1.58	0.05	1.61	0.05	1.83	0.03
rs1109461 (45)	C/T	01	01	1.28	0.78	1.28	0.78	0.78	0.78	27	26	0.99	0.94	0.98	0.94	0.88	0.63
rs1518108 (46)	C/T	42	49	0.73	0.07	0.7	0.05	1.55	0.17	52	58	0.75	0.14	0.76	0.15	0.92	0.77

[a]Where $P<0.05$ for any of the tests, or SNPs previously reported as significant for *IL10*, *IL19*, and *IL20* SNP and HCV clearance.
[b]Allelic tests examine alleles individually, codominant codes genotypes as either 0, 1, or 2 rare alleles, and dominant codes genotypes as no (0) or any (1) rare alleles.
[c]Second nucleotide indicates the major allele in European Americans whose frequency is reported for both groups.
[d]Numbers correspond to those in Table 1.
Note: Cases were matched to controls by HIV status, gender, and geographic location in the two racial groups.

Table 3 Association between HCV clearance and selected genotypes in conditional logistic regression in two racial groups

	European Americans								*African Americans*							
	Haplotype frequency (N (%))		*Allelic*		*Codominant*		*Dominant*		*Haplotype Frequency N (%)*		*Allelic*		*Codominant*		*Dominant*	
Gene Haplotypes	*Clearance (n = 216)*	*Chronic (n = 490)*	*Odds ratio*	*P*	*Hazard ratio*	*P*	*Odds ratio*	*P*	*Clearance (n = 182)*	*Chronic (n = 366)*	*Odds ratio*	*P*	*Hazard ratio*	*P*	*Odds ratio*	*P*
IL10																
GCAAA	47 (23.0)	102 (22.3)	0.97	0.87	1.03	0.89	1.08	0.76	74 (43.0)	135 (38.1)	1.22	0.28	1.2	0.35	1.41	0.23
AA<u>AC</u>A	1 (.5)	2 (.4)	0.78	0.84	0.78	0.84	0.78	0.84	10 (5.8)	34 (9.6)	0.51	0.07	0.54	0.11	0.55	0.13
GA<u>AC</u>A	1 (.5)	0 (–)	—	—	—	—	—	—	5 (2.9)	7 (2.0)	1.43	0.54	1.67	0.4	1.67	0.4
AC<u>AC</u>A	1 (.5)	0 (–)	—	—	—	—	—	—	3 (1.7)	8 (2.3)	1.0	1.0	1.0	1.0	1.0	1.0
GC<u>AC</u>A	54 (26.5)	130 (28.4)	0.93	0.73	0.83	0.37	0.81	0.4	24 (14.0)	38 (1.7)	1.32	0.33	1.25	0.44	1.47	0.24
AA<u>GC</u>A	1 (.5)	0 (–)	—	—	—	—	—	—	2 (1.2)	12 (3.4)	0.32	0.14	0.31	0.14	0.31	0.14
GA<u>GC</u>A	22 (1.8)	46 (1.0)	1.12	0.68	1.16	0.62	1.1	0.76	15 (8.7)	26 (7.3)	1.12	0.73	1.15	0.69	1.28	0.5
GC<u>GC</u>A	19 (9.3)	46 (1.0)	1.09	0.77	0.9	0.74	0.9	0.74	20 (11.6)	30 (8.5)	1.52	0.16	1.59	0.15	1.56	0.21
AA<u>GC</u>G	53 (26.0)	120 (26.2)	0.91	0.62	1.04	0.86	1.19	0.52	13 (7.6)	59 (16.7)	0.48	0.01	0.47	0.02	0.48	0.03
AC<u>GC</u>G	3 (1.5)	8 (1.7)	1.63	0.44	1.27	0.73	1.79	0.48	4 (2.3)	0 (–)	—	—	—	—	—	—
IL19/IL20																
CCAAAC	124 (57.4)	287 (58.8)	0.93	0.66	0.94	0.71	1.56	0.20	87 (47.8)	162 (44.3)	1.19	0.34	1.22	0.33	1.28	0.39
CCAGGT	3 (1.4)	4 (.8)	3.0	0.23	3.0	0.23	3.0	0.23	1 (.5)	2 (.5)	2.0	0.62	2.0	0.62	2.0	0.62
CTGAAC	32 (14.8)	83 (17.0)	0.94	0.78	0.92	0.74	0.8	0.42	21 (11.5)	67 (18.3)	0.59	0.05	0.59	0.05	0.56	0.04
TCAAAC	2 (.9)	8 (1.6)	0.67	0.62	0.67	0.62	0.67	0.62	0 (–)	1 (0.3)	—	—	—	—	—	—
TCAGGC	19 (8.8)	43 (8.8)	0.89	0.69	0.89	0.69	0.89	0.71	33 (18.1)	36 (9.8)	1.93	0.01	2.17	0.01	2.7	0.002
TCAGGT	31 (14.4)	58 (11.9)	1.17	0.53	1.15	0.56	1.17	0.58	6 (3.3)	15 (4.1)	0.79	0.64	0.78	0.63	0.78	0.63

Allelic tests examine alleles individually, codominant codes genotypes as either 0, 1, or 2 rare alleles, and dominant codes genotypes as no (0) or any (1) rare alleles. *IL10* haplotypes were estimated using the ordered SNPs: rs3024498, rs1800872, rs1800896, rs6693899, and rs6703630. The *IL10* established promoter haplotypes, defined by rs1800896 (*IL10* −1082) and rs1800872 (*IL10*−592), are underlined: <u>ATA</u>, <u>ACC</u>, and <u>GCC</u>. *IL19/IL20* haplotypes were built using the following ordered SNPs: rs2243191, rs1400986, rs3024517, rs2981573, and rs2232360.
Note: Cases are matched to controls by HIV status, gender, and geographic location. We used SNP loci where allelic association results were significant with $P<0.05$ or previously reported as significant in the literature for *IL10*. Haplotype frequencies (%) are based on the *N* chromosomes sampled from *n* individuals in each gene region.

Table 4 Association with the HCV clearance with proximal and distal haplotypes in the *IL10* promoter region in African Americans

	Haplotype frequency		*Allelic*		*Codominant*		*Dominant*	
Haplotypes	*Clearance (n (%))*	*Chronic (n (%))*	*Odds ratio*	*P*	*Hazard ratio*	*P*	*Odds ratio*	*P*
Proximal haplotypes[46]								
GCC	52 (30.8)	125 (35.7)	0.84	0.38	0.82	0.32	0.89	.64
ACC	42 (24.9)	88 (25.1)	0.96	0.85	0.97	0.91	1.07	0.93
ATA	75 (44.4)	137 (39.1)	1.22	0.31	1.16	0.44	1.3	0.44
Distal haplotypes[41]								
AAA	15 (8.9)	73 (20.9)	0.38	0.002	0.38	0.002	0.38	0.003
AGA	11 (6.5)	24 (6.9)	0.96	0.9	0.96	0.9	1.05	0.89
AGC	3 (1.8)	3 (.9)	2.0	0.4	2.0	0.4	2.0	0.4
TAA	10 (5.9)	29 (8.3)	0.7	.34	0.7	0.34	0.73	0.44
TAC	3 (1.8)	8 (2.3)	1.0	1.0	1.0	1.0	1.0	1.0
TGA	8 (4.7)	9 (2.6)	1.79	0.24	1.79	0.24	2.26	0.14
TGC	119 (70.4)	204 (58.3)	1.52	0.04	1.52	0.04	2.55	0.03

Proximal haplotypes include SNPs at positions −1082 (rs1800896), −819(rs1800871), and −592 (rs1800872) relative to the transcription start site; distal haplotypes include SNPs at positions −3575 (rs1800890), −2849 (rs6703630), and −2763 (rs6693899) relative to the transcription start site.

LD, hold promise,[63] especially because of the much lower rate of clearance in African Americans compared to European Americans.[7] While the incidence of newly acquired hepatitis C infection has diminished in the United States, HCV infection remains a high-priority problem that needs to be addressed utilizing many different approaches. In this sense, discovery of genes associated with the HCV clearance is a crucial step that can result in novel strategies for patient treatment and recovery. The results of this study could have implications for the identification of HCV clearance mechanisms as well as therapy decisions.

Methods

Study design

A total of 274 African Americans (91 clearance cases and 183 chronically infected matched controls) and 353 European Americans (108 clearance cases and 245 chronic infected matched controls) were chosen for a nested case–control study design. Individuals were selected from the ALIVE, MHCS, and HGDS cohorts for HIV-1/AIDS.[20] Case subjects were clear of viremia without any HCV-specific treatment, demonstrated by $\geq$2 instances separated by a minimum of 6 months, in which HCV RNA could not be detected in serum. Prior infection was substantiated by detection of HCV antibody (anti-HCV). Chronically infected control subjects selected from the same cohort had anti-HCV and HCV RNA in serum for $\geq$6 months. Control subjects were matched 2:1 to case subjects in the same cohort on the basis of HIV status, gender, geographic location, and race. These factors were chosen since HIV status and race are determinants of viral clearance in the ALIVE cohort.[7,13]

Genotyping

DNA was extracted from whole blood using the standard Qiagen protocol (Hilden, Germany) and by phenol–chloroform extraction.[64] SNP genotyping was performed by PCR on a Perkin Elmer Thermal Cycler 9700 (Foster City, CA, USA). All PCR reactions were performed using 10 ng DNA in a total volume of 10 µl. The PCR products were pooled together to make one master plate of all DNA. Up to 10 different DNA fragments were combined in a 384-well plate format. Subsets of the products were checked for amplification on agarose gels. We tested 54 SNPs in the *IL10* region (Figure 1), which included the genes *FNBP2, IKBKE, RASSF5, LGTN, DYRK3, MAPKAPK2*, *TOSO*, *PIGR*, *FKSG87*, and *SARG* along with *IL10* and its paralogs *IL19, IL20*, and *IL24*, for an association with HCV clearance *vs* persistence during infection. A total of 24 SNPs were sampled within *IL10*, and 30 more from the surrounding $\pm$300 kb region (Table 1).

SNPs were genotyped using two different methods: a multiplexed length-modified single base extension (SBE) and TaqMan. A length-modified SBE protocol from Applied Biosystems (AB, Foster City, CA, USA) was used for genotyping SNPs. Exonuclease I and shrimp alkaline phosphatase (SAP) enzymes were used to clean the amplification products, and SBE was performed using the SNaPshot Kit made by AB (Foster City, CA, USA),[65,66] which attaches a fluorescently labeled ddNTP to the extension primer. An additional SAP clean-up was performed to modify unincorporated ddNTPs using SAP. The reaction was added to a combination of formamide and GS-120 LIZ™ marker for size separation-based genotyping on the AB 3100 Genetic Analyzer. In addition, some genotyping was performed using TaqMan assay-by-design technology. Reactions were carried out in 384-well format with a total volume of 5 µl. The reactions were amplified using AB 9700 PCR machines and fluorescence was quantified with an AB 7900. The scoring process was simplified with automation using Genotyper and GeneMapper for SBE, or SDS software for TaqMan (AB). Some cases and controls were not typed due to lack of suitable samples or genotyping failures.

Statistical analysis

All analyses were perfomred with SAS Version 8.2. software.[47] First, all loci were analyzed for LD

(SAS/Genetics, PROC ALLELE). Two historic haplotype groups were identified: *IL10* and *IL19/IL20* (Figure 2). Each locus was also tested for Hardy–Weinberg (H-W) equilibrium (SAS/Genetics, PROC ALLELE). Loci that deviated from the predictions of H-W equilibrium were eliminated from further analysis, as they were likely to originate from low-frequency genotyping errors or other technical difficulties. Allele frequencies were calculated for HCV clearance and chronically infected individuals for each SNP locus. Loci with minor allelic frequencies $<3\%$ in both racial groups were not analyzed further.

Each variable SNP was evaluated for its association with HCV clearance in two different racial groups, European Americans and African Americans, by a log likelihood ratio χ^2 test.[47] In the subsequent tests, those loci that were significantly associated with HCV ($P<0.05$) , or those previously reported to be associated with HCV clearance in the literature (-1082 and -592 at *IL10*), were evaluated further.

Three different models of inheritance were used for these analyses: dominant, codominant, and allelic. Each allele was tested independently using conditional logistic regression (CLR)[67,68] on the data, with cases and controls matched by HIV status, sex, geographic location, and race. We further tested for additive genotypic association using three possible genotypes in each locus to calculate hazard ratios (HRs). Finally, we combined rare homozygotes with the heterozygotes and tested them in the same category against the common homozygotes for the association with HCV. ORs were calculated using PROC PHREG[47] to examine the likelihood of having the persistent form of HCV infection when carrying a specific allele. For the convenience of presenting, in the text, we indicated the range of ORs and HRs across the three models tested for each SNP and haplotype. *P*-values of CLR analysis reflected the statistical significance of the above analyses.

An association between SNP haplotypes and HCV clearance was also evaluated. Haplotypes within the two haplotype block regions around *IL10* and *IL19/IL20* (Figure 2) were estimated using the expectation-maximization (EM) algorithm in PROC HAPLOTYPE (SAS/Genetics). Then, high-probability haplotypes with frequencies $>1\%$ were tested for an association with HCV clearance using CLR, first as alleles and then as diplotypes in an additive codominant model. Finally, a dominant model of inheritance was used whereby heterozygotes and homozygotes for each haplotype were combined into one category, and then tested against another category where this particular haplotype was absent.

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